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Properties of Poly(3-Hydroxybutyrate) Depolymerase from a Marine Bacterium, *Alcaligenes faecalis* AE122

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Alcaligenes faecalis **AE122 that used poly(3-hydroxybutyrate) (PHB) as a sole source of carbon was newly isolated from a coastal seawater sample. The strain required seawater for growth on PHB as well as in a nutrient broth, in which seawater could be replaced by an appropriate concentration of NaCl. PHB depolymerase was purified to homogeneity from the culture supernatant of *A. faecalis* AE122 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme consisted of a monomer subunit with a molecular mass of 95.5 kDa. The N-terminal amino acid sequence was GAWQNNLAGGFNKV. The dimeric and trimeric esters of 3-hydroxybutyrate were the main hydrolysis products of the purified enzyme. The enzyme was most active at pH 9.0 and 55°C and was inhibited by phenylmethylsulfonyl fluoride. Several cations in seawater greatly enhanced the enzyme activity.**

Microbial reserve polyesters, such as poly(3-hydroxybutyrate) (PHB) and other polyhydroxyalkanoates, are especially attractive for commercial exploitation of biodegradable plastics (1, 5). These polymers, including their moldings, are known to be degraded by microbial action in a natural environment. Several kinds of PHB-degrading bacteria have been isolated (3, 6, 10, 11, 18), and the extracellular PHB-degrading enzyme PHB depolymerase has been purified from the culture supernatants of some of these bacteria and characterized (11, 15, 18). PHB-degrading bacteria examined in detail were isolated mostly from soils, lake water, and activated sludge for waste treatment (11). The degradation of these polymers is known to occur also in a marine environment (7), but microbial and biochemical knowledge of the process is limited. When such polymers are widely used, they will inevitably have an impact on the marine as well as the terrestrial environment.

We isolated a bacterium, *Alcaligenes faecalis* AE122, from a seawater sample to characterize its PHB-degrading ability. We describe herein the characterization of the isolate and the unique properties of the PHB depolymerase.

MATERIALS AND METHODS

Media and cultivation. The seawater-PHB medium consisted of 5 g of PHB (Aldrich Chemical Co. Inc.), 2 g of $NaNO_3$, 2 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 1 g of KH_2PO_4, 1 ml of vitamin mixture (20), 500 ml of artificial seawater (20), and 500 ml of H_2O, pH 7.0: hereafter, medium containing this amount of seawater will be referred to as 50% seawater. Artificial seawater was used instead of natural seawater except for early steps in the bacterial isolation. Cultivation was done on a rotary shaker (100 rpm) at 30°C in a 300-ml Erlenmeyer flask containing 100 ml of the PHB medium with an inoculum from a slope culture. Seawater nutrient agar (pH 7.0), which consisted of 5 g of peptone, 1 g of yeast extract, and 15 g of agar in 1,000 ml of 50% seawater, was used for slope and plate cultures. For the preparation of PHB depolymerase, a bacterium was grown at 30°C for 48 h in a 2-liter shake flask containing 1 liter of medium of the following composition: 2 g of PHB, 1 g of NH_4Cl, 0.1 g of $FeCl_3 \cdot 6H_2O$, 750 ml of 66 mM potassium phosphate (pH 6.8), and 250 ml of seawater. Culture was done with 1% (vol/vol) inoculum at 30°C for 48 h on a reciprocal shaker (100 strokes per min). The preculture was shaken (230 strokes per min) at 30°C for 48 h in a test tube (18 by 180 mm) containing 5 ml of succinate medium in which the PHB in the above medium was replaced by sodium succinate (5 g/liter) (18).

Isolation of PHB-degrading bacteria from seawater samples. PHB-degrading bacteria were isolated from seawater samples collected at the coast around Tottori Prefecture in Japan. Bacteria were isolated by selective enrichment in the seawater-PHB medium. Bacterial cells suspended in sterile 50% seawater containing 10% (vol/vol) glycerol were stored frozen at −80°C in plastic vials, in which most isolates survived at least 1 year.

Analyses. Growth was routinely monitored by measurement of the optical density at 650 nm. The test tubes were left to stand for 10 min before growth was measured so that residual PHB could accumulate at the bottom of the tubes. Protein was measured with a protein assay kit (Japan Bio-Rad Laboratories, Tokyo), with bovine serum albumin as the standard. PHB was degraded to 3-hydroxybutyrate methyl ester by acid methanolysis, which was determined by gas chromatography according to the method Braunegg et al. (2). Products of enzymatic hydrolysis of PHB were trimethylsilated, and their presence was determined by gas chromatography (12). Polyacrylamide (7%) gel electrophoresis (PAGE) was done at pH 9.5 according to the method of Davis (4). Sodium dodecyl sulfate (SDS)-PAGE was done by the method of Laemmli (14). The molecular weight of the enzyme was measured by high-pressure liquid chromatography on G-3000SW (TOSOH, Tokyo, Japan) (13). The amino acid sequence of the enzyme was determined by the Edman method with a Shimadzu PPSQ-10 protein sequencer.

Determination of PHB in liquid culture broth. Because the denatured preparation of PHB (a commercial product) is insoluble in water and hard to extract with organic solvents, it is difficult to trace PHB degradation quantitatively during cultivation. We devised a simple method for measurement of the residual PHB in culture broth. Screw-cap test tubes (15 by 150 mm) that contained 2 ml of PHB medium were shaken (230 strokes per min) at 30°C; a cotton plug was used during culture instead of the screw cap. One of the tubes was taken out after an appropriate time, and 0.1 ml of broth was used for a viable cell count on seawater nutrient agar. The residual broth in the test tube was centrifuged at 6,000 × *g* for 10 min. The supernatant was used for analysis of water-soluble degradation products. The precipitate in the test tube was directly lyophilized, and PHB in the resultant powder was detected by the method of Braunegg et al. (2).

Enzyme assay. PHB depolymerase activity was routinely assayed by the decrease in turbidity with PHB according to the method of Tanio et al. (18). PHB for the enzyme assay was extracted from cells of *Alcaligenes eutrophus* ATCC 17699 with sodium hypochlorite solution and then washed successively with water, ethanol, acetone, and ether, according to the method of Fukui et al. (9). A stable suspension of the PHB preparation in a buffer solution was obtained by treatment with a sonic oscillator (19 kHz, 10 min) (9). The standard reaction mixture contained 25 mM Tris-HCl (pH 7.5), 80 μg of PHB per ml, 50% seawater, and an appropriate amount of enzyme, in a total volume of 3.0 ml. The reaction was done at 30°C without shaking. One unit of enzyme activity was defined as the degradation of 1 μg of PHB per min (11). K_m and V_{max} were determined by using reciprocal plots of reaction velocity (micrograms per milliliter per milligram per minute) versus PHB concentration (micrograms per milliliter).

Enzyme purification. All manipulations were done at 0 to 5°C. *A. faecalis* AE122 culture broth was centrifuged at 17,000 × *g* for 40 min. The resultant clear supernatant (2.4 liters) was mixed with a 250-ml slurry of DEAE-cellulose DE52 (Whatman) that had been equilibrated with 50 mM Tris-HCl (pH 7.5) and then stirred gently at 4°C for 1 h. The DE52 slurry was packed into a glass column (5 by 12.7 cm) and then washed with a 5× volume (1,250 ml) of the equilibrated buffer. The enzyme was eluted with 750 ml of 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl. The active fractions (110 ml), to which solid NaCl had been

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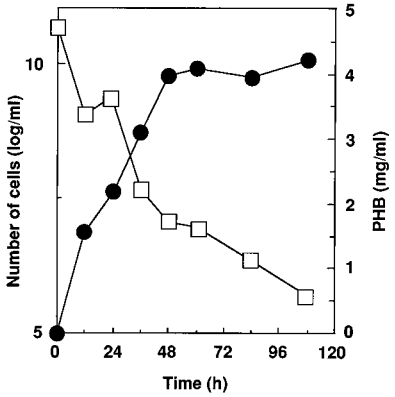

FIG. 1. Use of PHB by *A. faecalis* AE122. Medium containing PHB was used, and concentration of residual PHB (□) and the number of viable cells (●) were determined as described in Materials and Methods. The increase in cell number was negligible when PHB was omitted from the medium.

added to a concentration of 4 M, were put on a phenyl-Sepharose CL-4B column (2.5 by 6 cm) equilibrated with the 50 mM Tris-HCl (pH 7.5) containing 4 M NaCl. Elution was done with a decreasing NaCl concentration and an increasing ethylene glycol concentration gradient: the final concentrations were 0 M and 50%, respectively, the total elution volume being 200 ml. The pooled active solution (39 ml) was dialyzed against 1.5 liters of 50 mM Tris-HCl (pH 7.5) and chromatographed on a DEAE-Toyopearl pack 650 column (2.2 by 20 cm) which had been equilibrated with the same buffer. The elution was done with 230 ml of 50 mM Tris-HCl (pH 7.5) containing 0.8 M NaCl and then with linear increasing concentrations of NaCl in the buffer (0.8 to 1 M; total volume, 460 ml). The active fractions were pooled, dialyzed against 50 mM Tris-HCl (pH 7.5), and stored at 4°C until use.

Determination of enzyme products. The reaction mixture containing 1.0 mg of PHB, 25 mM Tris-HCl (pH 7.5), 50% seawater, and 2.0 μg of the purified enzyme in 2.5 ml was incubated at 30°C. The reaction was terminated by the addition of HCl to pH 2.0, and the hydrolyzed products were extracted with ethyl acetate. Ethyl acetate was removed under reduced pressure to give an oil. A portion of the oil was dissolved in 1,4-dioxane and directly used in gas chromatography under the following conditions: capillary column, J&W Scientific DB-5 (0.261 mm by 30 m); carrier gas, N_2; column temperature, 150°C for 5 min, with temperature increases at 10°C/min, and then 250°C for 15 min; injection temperature, 250°C. The electron ionization (EI) and chemical ionization (CI) mass spectra (MS) were run on a Hewlett-Packard model 5890 spectrometer equipped with a 5970B mass detector system or a Hitachi M2000 mass detector system, respectively.

RESULTS AND DISCUSSION

Isolation of PHB-degrading bacteria from seawater samples. A bacterial strain, AE122, that used PHB as a carbon source was isolated from a seawater sample collected in the harbor of Tottori, Japan. The principal bacteriological characteristics of the strain were as follows: gram-negative, short rod; no spores; motile; catalase and oxidase positive; and no fermentation in the glucose oxidation-fermentation test. The maximum growth temperature of strain AE122 was 45°C. Further characterization was carried out at the National Collection of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland, which demonstrated strain AE122 to be *A. faecalis.*

Degradation and utilization of PHB by the isolates. Figure 1 shows the course of PHB degradation by *A. faecalis* AE122. It became feasible to monitor the process of bacterial growth quantitatively on PHB in liquid culture by a convenient method, as described in Materials and Methods. The growth of the isolate was dependent on PHB degradation. The PHB in the culture broth was completely consumed within 7 days (data not shown). Bacterial metabolites from PHB, such as 3-hydroxybutyrate, the oligomers, and small-molecular-mass compounds that were extracted with ethyl acetate in an acidic pH and detected by gas chromatography, were not accumulated in broth cultured for 7 days. The pH of broth was lowered only to 6.2 to 6.5 throughout the culture. The isolate grew well on the monomer, dimer, and trimer of 3-hydroxybutyrate (the dimer and trimer were products of PHB depolymerase, as mentioned below). These findings indicate that the isolate can degrade PHB with little accumulation of degradation intermediates in the medium.

TABLE 1. Purification of PHB depolymerase from *A. faecalis* AE122

Purification step	Total protein (mg)	Total activity (kU)	Sp act (kU/mg)	Yield (%)	Purification (fold)
Culture supernatant	158.0	231	2.00	100	1
DEAE-cellulose (DE52)	8.5	212	25.0	92	13
Phenyl-Sepharose CL-4B	4.1	84.0	27.0	36	14
DEAE-Toyopearl	2.1	62.0	30.0	27	15

A. faecalis AE122 also grew on other polyhydroxyalkanoates, i.e., poly(3-hydroxybutyrate-*co*-[12%]3-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-[9%]4-hydroxyvalerate) (8). Growth was fastest on poly(3-hydroxybutyrate-*co*-[9%]4-hydroxyvalerate), followed by PHB and then poly(3-hydroxybutyrate-*co*-[12%]3-hydroxyvalerate).

Seawater requirement for growth. *A. faecalis* AE122 required essentially the salts in seawater for growth on PHB medium. The highest cell yield appeared in media containing 45 to 60% seawater. Seawater could be replaced by NaCl for growth but not by LiCl, KCl, $MgCl_2$, $CaCl_2$, or sucrose, suggesting that the Na^+ ion is essential for growth. The bacterium grew well at an NaCl concentration of 0.18 M, which is nearly the same NaCl concentration as in 45% seawater. The extent of growth in medium containing seawater was higher than that in NaCl. This means that the growth was stimulated by other salts in seawater. Seawater was required also for growth on nutrient broth containing 0.2% polypeptone, 0.05% yeast extract, and 0.1% K_2HPO_4, and the optimum concentrations of seawater and NaCl were 50% and 0.2 M, respectively. The requirement of seawater or the Na^+ ion is one of the most important physiological criteria in the identification of a bacterium of marine origin (19). In conclusion, the isolate is most probably an inhabitant of a marine environment.

Purification, molecular mass, and amino acid sequence of PHB depolymerase. PHB depolymerase was found in the cul-

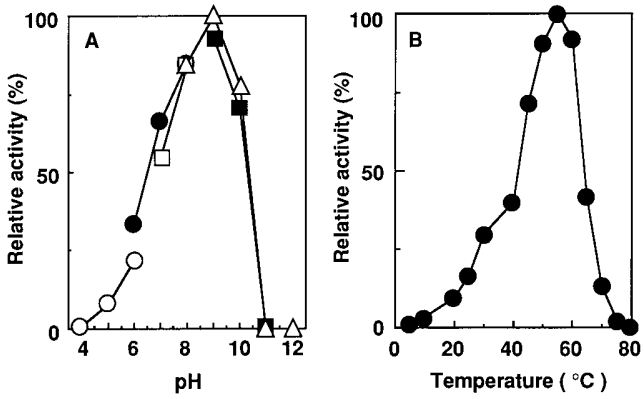

FIG. 2. Effect of pH (A) and temperature (B) on PHB depolymerase activity. (A) Buffer consisted of the following (50 mM each): ○, acetate; ●, potassium phosphate; □, Tris-HCl; △, glycine-NaOH; ■, borate-NaOH. (B) The activity was assayed under standard conditions at various temperatures.

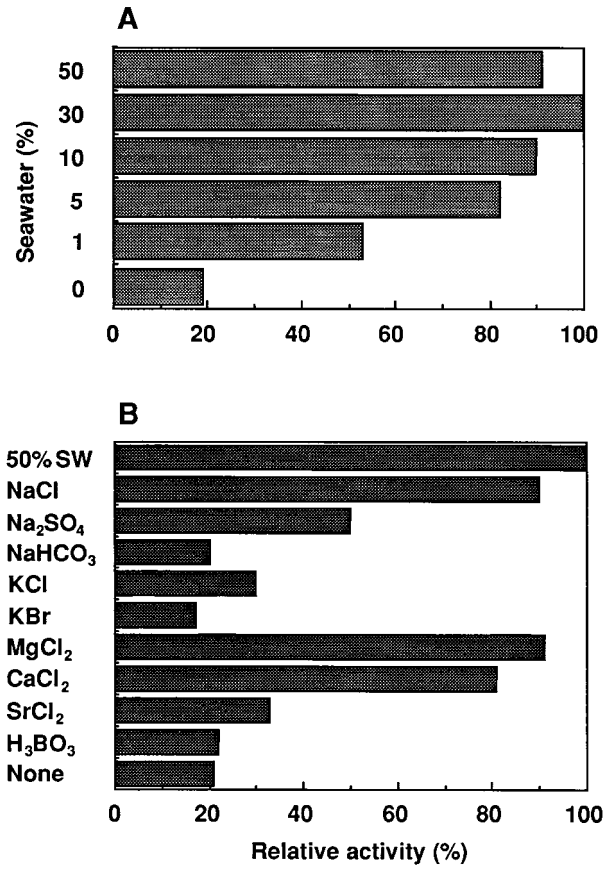

FIG. 3. Effect of seawater on PHB depolymerase activity. (A) The enzyme activity was assayed at 30°C in the standard reaction mixture containing various concentrations of seawater. (B) The reaction was carried out under standard conditions in which 50% seawater (SW) was replaced by one of the following salts (millimolar): NaCl (200), Na_2SO_4 (14), $NaHCO_3$ (0.85), KCl (4.4), KBr (0.42), $MgCl_2$ (12), $CaCl_2$ (3.8), $SrCl_2$ (0.045), or H_3BO_3 (0.24).

ture supernatants of *A. faecalis* AE122 and purified by the procedures described in Materials and Methods (Table 1). The purified enzyme gave one single band on PAGE and SDS-PAGE. The molecular masses of the native and SDS-treated enzymes were 90.2 and 95.5 kDa, respectively. The N-terminal amino acid sequence of the purified enzyme was GAWQNN LALGGFNKV. The enzyme is probably a monomeric protein with a relatively high molecular mass.

Products of PHB depolymerase reaction. Two distinct peaks were detected in the direct analysis of the products by gas chromatography (see Materials and Methods): peak I, MS (EI, 70 eV) *m/z* 175 $(M - 15)^+$, MS (CI, 70 eV) *m/z* 191 MH^+; peak II, MS (EI, 70 eV) *m/z* 261 $(M - 15)^+$, MS (CI, 70 eV) *m/z* 277 MH^+. The molecular masses of peaks I and II correspond to those of the dimeric and trimeric esters of 3-hydroxybutyrate, respectively, suggesting that these oligomers are the main products of the reaction with PHB depolymerase from *A. faecalis* AE122.

General properties of the enzyme. The enzyme was most active at pH 9.0 (Fig. 2A) and 55°C (Fig. 2B). The enzyme was completely inactivated by 1 mM phenylmethylsulfonyl fluoride, indicating that the activity is dependent on the serine residue of the enzyme protein. The activity was not affected by iodoacetate (an SH reagent), EDTA, *o*-phenanthroline, 2,2′-dipyridyl, or KCN (inhibitors of metalloenzymes) at 1 mM each.

Effect of metal salts on enzyme kinetics. The enzyme activity was greatly enhanced by the addition of seawater. The highest activity was found in the reaction mixture containing 30% seawater (Fig. 3A) and was not altered by an increase in seawater concentration from 50 to 100%. Figure 3B shows the activity in the reaction mixture containing each salt in the same concentration as in 50% seawater. With the addition of NaCl, $MgCl_2$, or $CaCl_2$, the activity was greatly stimulated at each concentration. The activity was dependent on the concentration of each salt (data not shown). Alkaline metal salts, NaCl, Na_2SO_4, and KCl, were required at relatively high concentrations (200 mM) for the same level of activity as in 50% seawater. Alkaline earth metal salts activated the enzyme at a much lower concentration (10 mM) than the alkaline metal salts, the effect being higher in the order $SrCl_2$, $CaCl_2$, and $MgCl_2$. The enzyme was barely affected by the addition of LiCl and completely inhibited by the addition of $Ba(CH_3COO)_2$ (10 mM) or $BeSO_4$ (0.1 mM). There was no distinct difference in the V_{max} (0.17 to 0.19 μg/ml/mg/min) obtained in the reaction mixtures containing 200 mM NaCl, 10 mM $SrCl_2$, and 50% seawater. On the other hand, the K_m values were greatly affected by the salts in the reaction mixture; K_ms were 270, 100, and 6.7 μg/ml in 200 mM NaCl, 10 mM $SrCl_2$, and 50% seawater, respectively. The K_m in 50% seawater was comparable to that of the enzyme from terrestrial strains (15, 18). These results suggest that the affinity of the enzyme of *A. faecalis* AE122 to PHB is highly enhanced by synergetic effects obtained by the different salts in seawater.

Table 2 gives some properties of the PHB depolymerase. The enzyme of *A. faecalis* AE122 released a dimer and trimer as the hydrolytic products of PHB and was inhibited by phenylmethylsulfonyl fluoride, results analogous to those obtained with the enzyme from a terrestrial strain, *A. faecalis* T1 (18). However, the N-terminal amino acid sequence, molecular weight of the enzyme, and optimum pH for activity of marine strain AE122 differ greatly from those of terrestrial strain T1. The enzyme of strain AE122 is different from that of *Coma-*

TABLE 2. Comparison of properties of PHB depolymerase from several bacteria

Bacterium (reference)	Molecular mass (kDa)	Subunit molecular mass (kDA)	Optimum temp (°C)	Optimum pH	Inhibitor	Product(s) of hydrolysis[a]	N-terminal amino acid sequence
A. faecalis AE122 (this work)	90	95	55	9.0	PMSF	Dimer, trimer	GAWQNNLALGGFNKV
A. faecalis T1 (18)	48–50	48		7.5	PMSF	Dimer, trimer	ATAGPGAWSSQETWA[b]
Comamonas sp. (11)	44–45	44	30	9.2	Dithioerythritol	Monomer	
P. lemoignei (15)[c]	45–49	54–58		8.0	PMSF, dithioeryritol	Monomer, dimer, trimer	

[a] Monomer, 3-hydroxybuyrate; dimer, dimeric ester; trimer, trimeric ester.
[b] From reference 16.
[c] PHB depolymerase from this strain was divided into four fractions by carboxymethyl cellulose.

monas sp. (11) in many properties, except pH-dependent activity, and from that of *Pseudomonas lemoignei* (15) in molecular mass and hydrolysis pattern.

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